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FIBER OPTIC SENSOR WITH ENCODED MICROSPHERES

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BACKGROUND OF THE INVENTION

The use of optical fibers and optical fiber strands in combination with light absorbing dyes for chemical
5 analytical determinations has undergone rapid development, particularly within the last decade. The use of optical fibers for such purposes and techniques is described by Milanovich et al., "Novel Optical Fiber Techniques For Medical Application", Proceedings of the SPIE 28th Annual
10 International Technical Symposium On Optics and Electro-Optics, Volume 494, 1980; Seitz, W.R., "Chemical Sensors Based On Immobilized Indicators and Fiber Optics" in *C.R.C. Critical Reviews In Analytical Chemistry*, Vol. 19, 1988, pp. 135-173; Wolfbeis, O.S., "Fiber Optical Fluorosensors
15 In Analytical Chemistry" in *Molecular Luminescence Spectroscopy, Methods and Applications* (S. G. Schulman, editor), Wiley & Sons, New York (1988); Angel, S.M., *Spectroscopy 2* (4):38 (1987); Walt, et al., "Chemical Sensors and Microinstrumentation", *ACS Symposium Series*,
20 Vol. 403, 1989, p. 252, and Wolfbeis, O.S., *Fiber Optic Chemical Sensors*, Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume.

When using an optical fiber in an *in vitro/in vivo* sensor, one or more light absorbing dyes are located near
25 its distal end. Typically, light from an appropriate source is used to illuminate the dyes through the fiber's proximal end. The light propagates along the length of the optical fiber; and a portion of this propagated light exits the

distal end and is absorbed by the dyes. The light absorbing
dye may or may not be immobilized; may or may not be
directly attached to the optical fiber itself; may or may
not be suspended in a fluid sample containing one or more
5   analytes of interest; and may or may not be retainable for
subsequent use in a second optical determination.

Once the light has been absorbed by the dye, some
light of varying wavelength and intensity returns, conveyed
through either the same fiber or collection fiber(s) to a
10 detection system where it is observed and measured. The
interactions between the light conveyed by the optical
fiber and the properties of the light absorbing dye provide
an optical basis for both qualitative and quantitative
determinations.

15   Of the many different classes of ligh: absorbing dyes
which conventionally are employed with bundles of fiber
strands and optical fibers for different analytical
purposes are those more common compositions that emit light
after absorption termed "fluorophores" and those which
20 absorb light and internally convert the absorbed light to
heat, rather than emit it as light, termed "chromophores."

Fluorescence is a physical phenomenon based upon the
ability of some molecules to absorb light (photons) at
specified wavelengths and then emit light of a longer
25 wavelength and at a lower energy. Substances able to
fluoresce share a number of common characteristics: the
ability to absorb light energy at one wavelength λ_{ab}; reach
an excited energy state; and subsequently emit light at
another light wavelength, λ_{em}. The absorption and
30 fluorescence emission spectra are individual for each
fluorophore and are often graphically represented as two

separate curves that are slightly overlapping. The same
fluorescence emission spectrum is generally observed
irrespective of the wavelength of the exciting light and,
accordingly, the wavelength and energy of the exciting
5   light may be varied within limits; but the light emitted by
the fluorophore will always provide the same emission
spectrum. Finally, the strength of the fluorescence signal
may be measured as the quantum yield of light emitted. The
fluorescence quantum yield is the ratio of the number of
10   photons emitted in comparison to the number of photons
initially absorbed by the fluorophore. For more detailed
information regarding each of these characteristics, the
following references are recommended: Lakowicz, J. R.,
Principles of Fluorescence Spectroscopy, Plenum Press, New
15   York, 1983; Freifelder, D., Physical Biochemistry, second
edition, W. H. Freeman and Company, New York, 1982;
"Molecular Luminescence Spectroscopy Methods and
Applications: Part I" (S.G. Schulman, editor) in *Chemical
Analysis*, vol. 77, Wiley & Sons, Inc., 1985; *The Theory of
20   Luminescence*, Stepanov and Gribkovskii, Iliffe Books, Ltd.,
London, 1968.

In comparison, substances which absorb light and do
not fluoresce usually convert the light into heat or
kinetic energy. The ability to internally convert the
25   absorbed light identifies the dye as a "chromophore." Dyes
which absorb light energy as chromophores do so at
individual wavelengths of energy and are characterized by a
distinctive molar absorption coefficient at that
wavelength. Chemical analysis employing fiber optic
30   strands and absorption spectroscopy using visible and
ultraviolet light wavelengths in combination with the
absorption coefficient allow for the determination of
concentration for specific analyses of interest by spectral
measurement. The most common use of absorbance measurement

via optical fibers is to determine concentration which is calculated in accordance with Beers' law; accordingly, at a single absorbance wavelength, the greater the quantity of the composition which absorbs light energy at a given
5   wavelength, the greater the optical density for the sample. In this way, the total quantity of light absorbed directly correlates with the quantity of the composition in the sample.

Many of the recent improvements employing optical
10   fiber sensors in both qualitative and quantitative analytical determinations concern the desirability of depositing and/or immobilizing various light absorbing dyes at the distal end of the optical fiber. In this manner, a variety of different optical fiber chemical sensors and
15   methods have been reported for specific analytical determinations and applications such as pH measurement, oxygen detection, and carbon dioxide analyses. These developments are exemplified by the following publications: Freeman, et al., *Anal Chem.* 53:98 (1983); Lippitsch et al.,
20   *Anal. Chem. Acta.* 205:1, (1988); Wolfbeis et al., *Anal. Chem.* 60:2028 (1988); Jordan, et al., *Anal. Chem.* 59:437 (1987); Lubbers et al., *Sens. Actuators* 1983; Munkholm et al., *Talanta* 35:109 (1988); Munkholm et al., *Anal. Chem.* 58:1427 (1986); Seitz, W. R., *Anal. Chem.* 56:16A-34A
25   (1984); Peterson, et al., *Anal. Chem.* 52:864 (1980): Saari, et al., *Anal. Chem.* 54:821 (1982); Saari, et al., *Anal. Chem.* 55:667 (1983); Zhujun et al., *Anal. Chem. Acta.* 160:47 (1984); Schwab, et al., *Anal. Chem.* 56:2199 (1984); Wolfbeis, O.S., "Fiber Optic Chemical Sensors", *Ed. CRC*
30   *Press, Boca Raton, FL,* 1991, 2nd Volume; and Pantano, P., Walt, D.R., *Anal. Chem.,* 481A-487A, Vol. 67, (1995).

More recently, fiber optic sensors have been constructed that permit the use of multiple dyes with a single, discrete fiber optic bundle. U.S. Pat. Nos. 5,244,636 and 5,250,264 to Walt, *et al.* disclose systems

5   for affixing multiple, different dyes on the distal end of the bundle, the teachings of each of these patents being incorporated herein by this reference. The disclosed configurations enable separate optical fibers of the bundle to optically access individual dyes. This avoids the

10 problem of deconvolving the separate signals in the returning light from each dye, which arises when the signals from two or more dyes are combined, each dye being sensitive to a different analyte, and there is significant overlap in the dyes' emission spectra.

15 SUMMARY OF THE INVENTION

The innovation of the two previous patents was the placement of multiple chemical functionalities at the end of a single optical fiber bundle sensor. This configuration yielded an analytic chemistry sensor that could be remotely

20 monitored via the typically small bundle. The drawback, however, was the difficulty in applying the various chemistries associated with the chemical functionalities at the sensor's end; the functionalities were built on the sensor's end in a serial fashion. This was a slow process,

25 and in practice, only tens of functionalities could be applied.

The present design is based on two synergistic inventions: 1) the development of a bead-based analytic chemistry system in which beads, also termed microspheres,

30 carrying different chemical functionalities may be mixed together while the ability is retained to identify the functionality of each bead using an optically interrogatable encoding scheme; and 2) an optical fiber

bundle sensor in which the separate beads or microspheres may be optically coupled to discrete fibers or groups of fibers within the bundle. Each invention has separate applications but, when implemented together, yields an
5 optical fiber sensor that can support large numbers, thousands or more, of separate chemical functionalities, which is relatively easy to manufacture and use.

In general, according to one aspect, the invention concerns an analytic chemistry system that comprises a
10 population of beads or microspheres. Within the population are separate subpopulations, each of which carries chemical functionality which changes an optical signature of the beads in a presence of targeted analytes. This signature change can occur via many different mechanisms. A few
15 examples include the binding of a dye-tagged analyte in the bead, the production of a dye species on or near the beads, the destruction of an existing dye species, a change in optical signal upon analyte interaction with dye on bead, or any other optical interrogatable event. Although the
20 subpopulations may be randomly mixed together, the chemical functionality on each bead is determined via an optical signature which is encoded with a description o f the chemical functionality. As a result, by observing whether the optical signature of a particular bead is
25 exhibiting a change, or not, and then decoding the signature for the functionality of the bead, the presence or not of the analyte targeted by the functionality may be determined.

In specific embodiments, the beads are encoded using
30 dyes that are preferably entrapped within the beads, the chemical functionality being added on surfaces. The dyes may be chromophores or phosphors but are preferably fluorescent dyes, which due to their strong signals provide

a good signal-to-noise ratio for decoding. The encoding can be accomplished in a ratio of at least two dyes, although more encoding dimensions may be added in the size of the beads, for example.

5   According to another aspect, the invention also concerns an analytic chemistry fiber optic bundle sensor. This sensor has a population of beads carrying chemical functionalities at, on or near, a distal end of the bundle. The ability to monitor optical signature changes associated

10   with individual or multiple beads is provided by coupling those signature changes into separate optical fibers or groups of fibers of the bundle for transmission to the proximal end where analysis is performed either manually, by the user, or automatically, using image processing

15   techniques.

        In the preferred embodiment, each one of the beads is located within separate wells formed at terminal ends of optical fibers of the bundle. These microwells are formed by anisotropic etching of the cores of the optical fibers

20   with respect to the cladding.

        Also in the preferred embodiment, the population of beads includes separate subpopulations, typically randomly distributed in an array across the bundle end, each subpopulation carrying a different chemical functionality

25   and an optically interrogatable code descriptive of the chemical functionality.

        Although each sensor is different insofar that it has a different distribution of the subpopulations of beads within its wells, only those beads that exhibit a positive

30   optical response or signature change need to be decoded. Therefore, the burden is placed on the analysis rather than

on sensor manufacture. Moreover, since the beads and fibers in the array can be monodisperse, the fluorescent regions arising from signal generation are extremely uniform and can be analyzed automatically using commercially available
5   microscopy analysis software, such image processing software is capable of defining different spectral regions automatically and counting the number of segments within each region in several seconds.

10   The above and other features of the invention including various novel details of construction and combinations of parts, and other advantages, will now be more particularly described with reference to the accompanying drawings and pointed out in the claims. It will be understood that the particular method and device
15   embodying the invention are shown by way of illustration and not as a limitation of the invention. The principles and features of this invention may be employed in various and numerous embodiments without departing from the scope of the invention.

20   BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings, reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale; emphasis has instead been placed upon illustrating the principles of the
25   invention. Of the drawings:

Fig. 1 is a schematic diagram illustrating the optical signature encoding and chemical functionalizing of the microspheres according to the present invention;

Fig. 2 is a process diagram describing the
30   preparation, encoding, and functionalizing of the microspheres of the present invention;

Fig. 3 is a schematic diagram illustrating a microsphere system including microspheres with different

chemical functionalities and encoded descriptions of the functionalities;

Fig. 4 is a schematic diagram of the inventive fiber optic sensor and associated instrumentation and control
5 system;

Fig. 5A and 5B are micrographs illustrating the preferred technique for attaching or affixing the microspheres to the distal end of the optical fiber bundle;

Fig. 6 is a process diagram describing well formation
10 in the optical fiber bundle and affixation of the microspheres in the wells;

Figs. 7A and 7B are micrographs showing the array of microspheres in their corresponding wells prior and subsequent to physical agitation, tapping and air pulsing,
15 demonstrating the electrostatic binding of the microspheres in the wells;

Figs. 8A, 8B, and 8C are mlcrographs from alkaline phosphatase microspheres when exposed to fluorescein diphosphate, at the fluorescein emission wavelength, at an
20 encoding wavelength for DiIC, and at an encoding wavelength for TRC, respectively;

Figs. 9A and 9B are micrographs showing the optical signal from β-galactosidase microspheres when exposed to fluorescein β-galactopyranoside at the fluorescein emission
25 wavelength and at an encoding wavelength for DiIC, respectively; and

Fig. 10A and 10B are micrographs showing the optical response from xrabbit antibody microspheres prior to and post, respectively, exposure to fluorescein labeled
30 antigens.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
1. Microspheres:

Fig. 1 illustrates the construction of a bead or microsphere 10 according to the principles of the present

invention. In common with the prior art, the microsphere 10 is given a chemical functionality 12, which is typically applied to the microsphere's surface. The chemical functionality is designed so that in the presence of the analyte(s) to which it is targeted, an optical signature of the microsphere, possibly including region surrounding it, is changed.

The bead or microsphere need not be spherical, irregular beads may be used. They are typically constructed from plastic or ceramic, and bead sizes ranging from nanometers, e.g., 500 nm, to millimeters, e.g., 1 mm, may be used. Moreover, various bead chemistries may be used. Beads or microspheres comprising methylstyrene, polystyrene, acryic polymer, latex, paramagnetic, thoria sol, carbon graphityed, and titanium dioxide are various possibilities. "Microsphere Detection Guide" from Bangs Laboratories, Fishers IN. is a helpful guide.

The inventive microsphere has an optical signature that is encoded with a description of the chemical functionality. In the preferred embodiment, reporter dyes 14 are added to the microsphere 10 with the encoding occurring in the ratio of two or more dyes. The reporter dyes 14 may be chromophore-type. Fluorescent dyes, however, are preferred because the strength of the fluorescent signal provides a better signal-to-noise ratio when decoding. Additionally, encoding in the ratios of the two or more dyes, rather than single dye concentrations, is preferred since it provides insensitivity to the intensity of light used to interrogate the reporter dye's signature and detector sensitivity.

Fig. 2 is a process diagram illustrating the preparation of the microspheres. In step 50, an aliquot of stock microspheres are vacuum filtered to produce a dry cake. In one implementation, microspheres of methylstyrene

5 (87%) and divinylbenzene (13%) are used that have a 3.1 micrometer (μm) diameter.

The microspheres may be purchased with the desired chemical functionalities already present. A large selection of such pre-prepared microspheres are currently available

10 from a number of commercial vendors. Alternatively, "blank" microspheres may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Some examples of these surface chemistries for blank microspheres are listed in Table I.

15   Table I

Surface chemistry	Name:
NH_2	Amine
COOH	Carboxylic Acid
CHO	Aldehyde
CH_2-NH_2	Aliphalic Amine
$CO\ NH_2$	Amide
CH_2-Cl	Chloromethyl
$CONH-NH_2$	Hydrazide
OH	Hydroxyl
SO_4	Sulfate
SO_3	Sulfonate
$Ar\ NH_2$	Aromatic Amine

The dry cake is then broken apart and a dye solution added to it in step 52 to encode optical signatures of the

30 microspheres with information concerning the intended

surface chemical functionalities. Dyes may be covalently bonded to the microspheres' surface, but this consumes surface binding sites desirably reserved for the chemical functionalities. Preferably, the microspheres are placed in a dye solution comprising a ratio of two or more fluorescent reporter dyes dissolved in an organic solvent that will swell the microspheres, e.g., dimethylformamide (DMF). The length of time the microspheres are soaked in the dye solution will determine their intensity and the broadness of the ratio range. Longer times yield higher intensities, but broader ratio ranges.

In an exemplary two dye system, Texas Red Cadaverine (TRC) is used, which is excited at λ_{ab}=580 mm and emits at λ_{em}=630 mm, in combination with indodicarbocyanine (DiIC): 610/670 ($\lambda_{ab}/\lambda_{em}$). Generally, dyes are selected to be compatible with the chemistries involved in the analysis and to be spectrally compatible. The emission wavelengths of the dyes should not overlap the regions of the optical spectrum in which the chemical functionalities induce changes in the microsphere signatures. This avoids deconvolution problems associated with determining signal contributions based on the presence of both the analyte and the encoding dye ratios contributing to an overlapping emission spectral region.

Examples of other dyes that can be used are Oxazin (662/705), IR-144 (745/825), IR-140 (776/882), IR-125 (786/800) from Exiton, and Bodipy 665/676 from Molecular Probes, and Naphthofluorescein (605/675) also from Molecular Probes. Lanthide may also be used. Fluorescent dyes emitting in other than the near infrared may also be used. Chromophore dyes are still another alternative that produce an optically interrogatable signature, as are more exotic formulations using Raman scattering-based dyes or

polarizing dyes, for example. The ability of a particular dye pair to encode for different chemical functionalities depends on the resolution of the ratiometric measurement. Conservatively, any dye pair should provide the ability to
5   discriminate at least twenty different ratios The number of unique combinations of two dyes made with a particular dye set is shown in the following Table II.

Table II

Number of dyes in set	Combinations possible
3	3
4	6
5	10
6	15

15 Thus, using six dyes and twenty distinct ratios for each dye pair, 300 separate chemical functionalities may be encoded in a given population of microspheres. Combining more than two dyes provides additional diversity in the encoding combinations.

20   In step 54, the microspheres are vacuum filtered to remove excess dye. The microspheres are then washed in water or other liquid that does not swell the microspheres, but in which the dyes are still soluble. This allows the residual dye to be rinsed off without rinsing the dye out
25 of the microspheres.

        In step 56, the chemical functionality is attached to the microsphere surface chemistries if not already present. It should be understood that surface chemistries may be present throughout the microsphere's volume, and not
30 limited to the physical circumferential surface.

In the prior art, a large spectrum of chemical functionalities have been manifest on microspheres that produce optically interrogatable changes in the presence of the targeted analyte. These functionalities include four
5 broad classifications of microsphere sensors: 1) basic indicator chemistry sensors; 2) enzyme-based sensors; 3) immuno-based sensors; and 3) geno-sensors.

A large number of basic indicator microsphere sensors have been previously demonstrated. Examples include:
10   Table III

TARGE ANALYTE	CHEMICAL FUNCTIONALITY	NOTES $(\lambda_{AB}/\lambda_{EM})$
pH Sensors based on:	seminaphthofluoresceins	e.g., carboxy-SNAFL
	seminaphthorhodafluors	e.g., carboxy-SNARF
	8-hydroxypyrene-1,3,6-trisulfonic acid	
	fluorescein	
CO2 Sensors based On:	seminaphthofluoresceins	e.g., carboxy-SNAFL
	seminaphthorhodafluors	e.g., carbody-SNARF
	8-hydroxypyrene-1,3,6-trisulfonic acid	
Metal Ions Sensors based on:	desferriozamine B	e.g., Fe
	cyclen derivative	e.g., Cu, Zn
	derivatized peptides	e.g., FITC-Gly-Gly-His, and FITC-Gly His, Cu, Zn
	fluorexon (calcine)	e.g., Ca, Mg, Cu, Pb, Ba
	calcine blue	e.g., Ca, Mg, Cu
	methyl calcine blue	e.g., Ca, Mg, Cu
	ortho-dianisidine tetracetic acid (ODTA)	e.g., Zn
	bis-salicylidene ethylenediamine (SED)	e.g., Al

	N-(6-methozy-8-quinolyl-p-toluenesulfonamine (TSQ)	e.g., Zn
	Indo-1	e.g., Mn, Ni
	Fura-2	e.g., Mn, Ni
	Magesium Green	e.g., Mg, Cd, Tb
O_2	Siphenylisobenzofuran	409/476
	Methoxyvinyl pyrene	352/401
Nitrite	diaminonaphthaline	340/377
NO	luminol	355/411
	dihydrohodamine	289/none
Ca^{2+}	Bis-fura	340/380
	Calcium Green	visible light/530
	Fura-2	340/380
	Indo-1	405/485
	Fluo-3	visible light/525
	Rhod-2	visible light/570
Mg^{2+}	Mag-Fura-2	340/380
	Mag-Fura-5	340/380
	Mag-Indo-1	405/485
	Magnesium Green	475/530
	Magnesium Orange	visible light/545
Zn^{2+}	Newport Green	506/535
TSQ	Methoxy-Quinobyl	334/385
Cu^+	Phen Green	492/517
Na^+	SBFI	339/565
	SBFO	354/575
	Sodium Green	506/535
K^+	PBFI	336/557
Cl^-	SPQ	344/443
	MQAE	350/460

Each of the chemicals listed in Table III directly produces an optically interrogatable signal or optical signature change in the presence of the targeted analyte.

Enzyme-based microsphere sensors have also been demonstrated and could be manifest on microspheres. Examples include:

Table IV

SENSOR TARGET	CHEMICAL FUNCTIONALITY
Glucose Sensor	glucose oxidase (enz.) + O_2-sensitive dye (see Table I)
Penicillin Sensor	penicillinase (enz.) + pH-sensitive dye (see Table I)
Urea Sensor	urease (enz.) + pH-sensitive dye (see Table I)
Acetylcholine Sensor	acetylcholinesterase (enz.) + pH-sensitive dye (see Table I)

Generally, the induced change in the optical signature due to the presence of the enzyme-sensitive chemical analyte occurs indirectly in this class of chemical functionalities. The microsphere-bound enzyme, e.g., glucose oxidase, decomposes the target analyte, e.g., glucose, consume a co-substrate, e.g., oxygen, or produce some by-product, e.g., hydrogen peroxide. An oxygen sensitive dye is then used to trigger the signature change.

Techniques for immobilizing enzymes on microspheres, are known in the prior art. In one case, NH_2 surface chemistry microspheres are used. Surface activation is achieved with a 2.5% glutaraldebyde in phosphate buffered saline (10 mM) providing a pH of 6.9. (138 mM NaCl, 2.7 mM, KCl). This is stirred on a stir bed for approximately 2 hours at room temperature. The microspheres are then rinsed with ultrapure water plus 0.01% tween 20 (surfactant) -0.02%, and rinsed again with a pH 7.7 PBS plus 0.01% tween

20. Finally, the enzyme is added to the solution, preferably after being prefiltered using a 0.45μm amicon micropure filter.

Immuno-based microsphere sensors have been

5 demonstrated for the detection for environmental pollutants such as pesticides, herbicides, PCB's and PAH's. Additionally, these sensors have also been used for diagnostics, such as bacterial (e.g., leprosy, cholera, lyme disease, and turburculosis), viral (e.g., HIV, herpes

10 simplex, cytomegalovirus), fungal (e.g., aspergillosis, candidiasis, cryptococcoses), Mycoplasmal (e.g., mycoplasmal pneumonia), Protozoal (e.g., amoebiasis, toxoplasmosis), Rickettsial (e.g., Rock Mountain spotted fever), and pregnancy tests.

15 Microsphere genosensors have also been demonstrated. These are typically constructed by attaching a probe sequence to the microsphere surface chemistry, typically via an NH_2 group. A fluorescent dye molecule, e.g., fluorescein, is attached to the target sequence, which is

20 in solution. The optically interrogatable signature change occurs with the binding of the target sequences to the microsphere. This produces a higher concentration of dye surrounding the microsphere than in the solution generally. A few demonstrated probe and target sequences, see

25 Ferguson, J.A. et al. *Nature Biotechnology*, Vol. 14, Dec. 19996, are listed below in Table V.

Table V

PROBE SEQUENCES	TARGET SEQUENCES
B-glo(+) (segment of human B-globin) 5'-NH$_2$-(CH$_2$)$_8$-)TT TTT TTT TCA ACT TCA TCC ACG TTC ACC-3	B-glo(+)-CF 5'-Fluorescein-TC AAC GTG GAT GAA GTT C-3'
IFNG(interferon gamma 1) 5'-NH$_2$-(CH$_2$)$_8$-T$_{12}$-TGG CTT CTC TTG GCT GTT ACT-3'	IFNG-CF 5'-Fluorescein-AG TAA CAG CCA AGA GAA CCC AAA-3'
IL2(interleukin-2) 5'-NH$_2$-(CH$_2$)$_8$-T$_{12}$-TA ACC GAA TCC CAA ACT CAC CAG-3'	IL2-CF 5'-Fluorescein-CT GGT GAG TTT GGG ATT CTT GTA-3'
IL4(interleukin-4) 5'NH$_2$-(CH$_2$)$_8$-T$_{12}$-CC AAC TGC TTC CCC CTC TGT-3'	IL4-CF 5'-Fluorescein-AC AGA GGG GGA AGC AGT TGG-3'
IL6(interleukin-6) 5'NH$_2$-(CH$_2$)$_8$-T12-GT TGG GTC AGG GGT GGT TAT T-3'	IL6-CF 5'-Fluorescein-AA TAA CCA CCC CTG ACC CAA C-3'

Alternatively, upon binding of the target sequences, an intercalating dye (e.g., ethidium bromide) can be added subsequently to signal the presence of the bound target to the probe sequence.

Fig. 3 illustrates the construction of an analytic chemistry system 100 from microspheres that have different chemical functionalities. Subpopulations of microspheres are represented as 10a, 10b, 10c carrying respective probe sequences 60a, 60b, 60c, as exemplary functionalities. These subpopulations, once manufactured, are combined and mixed with each other. The resulting combined population contains microspheres with randomly distributed individual chemical functionalities.

Typically, mixing microspheres with different
functionalities results in the loss of information
regarding the selectivity for each of the corresponding
target sequences. In a solution of microspheres with each
5   of the probe sequences 60a, 60b, and 60c, it is possible to
determine that at least one of the target sequences 62a,
62b, and 62c is present when a fluorescent marker dye 64
concentration is observed on the microspheres 10. There is
no way, however, to determine which probe sequence 60a,
10   60b, and 60c is generating the activity since the
information concerning which microsphere contained which
probe sequence was lost when the subpopulations were mixed.

In contrast, in the present invention, each
microsphere in each subpopulation is encoded with a common
15   optical signature. In the illustrated example, the
subpopulation represented by microsphere 10a has a two
reporter dye ratio of 10:1; the subpopulation of
microspheres 10b has a ratio of 1:1 of the same reporter
dyes, and subpopulation of microspheres 10c has a ratio of
20   1:10 of the reporter dyes.

Thus, the randomly mixed subpopulations of
microspheres are useful as an analytic chemistry based on
each of the carried chemical functionalities 60a-60c
separately. The 100 is added to an analyte of interest to
25   which some of the chemical functionalities may interact.
Any interaction changes the optical response of the
corresponding microspheres by, for example, binding a
fluorescent dye 64 to the microspheres. By identifying the
chemical functionalities of the microsphere in which the
30   optical signature has changed, using the encoded dye
combinations, information regarding the chemical identity
and concentration of the analyte may be gained based upon
the interaction or noninteraction of each functionality

contained in the probe 100.

    The microspheres exhibiting activity or changes in
their optical signature may be identified by utilizing a
somewhat "manual" approach of observing the individual
5 microspheres through a microscope. Decoding can also be
performed manually, depending on the particular reporter
dyes used. It may be helpful to use optical aids such as
light filters to observe the light from the microspheres at
emission wavelengths of the reporter dyes. While this
10 approach is possible, in the preferred embodiment, the
analytic chemistry microsphere is used with the inventive
optical fiber sensor.

2. Optical Fiber Sensor
    Fig. 4 is a schematic block diagram showing the
15 inventive optical fiber sensor 200 and associated control
210. The fiber optic sensor 200 comprises a fiber optic
bundle 202, that is constructed from separately clad fibers
so that light does not mix between fibers. The microsphere
100 is attached to the bundle's distal end 212, with the
20 proximal end 214 being received by a z-translation stage
216 and x-y micropositioner 218. These two components act
in concert to properly position the proximal end 214 of the
bundle 202 for a microscope objective lens 220. Light
collected by the objective lens 220 is passed to a
25 reflected light fluorescence attachment with three pointer
cube slider 222. The attachment 222 allows insertion of
light from a 75 Watt Xe lamp 224 through the objective lens
220 to be coupled into the fiber bundle 202. The light from
the source 224 iS condensed by condensing lens 226, then
30 filtered and/or shuttered by filter and shutter wheel 228,
and subsequently passes through a ND filter slide 230.

Light returning from the distal end 212 of the bundle
202 is passed by the attachment 222 to a magnification
changer 232 which enables adjustment of the image size of
the fiber's proximal or distal end. Light passing through
5   the magnification changer 232 is then shuttered and
filtered by a second wheel 234. The light is then imaged on
a charge coupled device (CCD) camera 236. A computer 238
executes imaging processing software to process the
information from the CCD camera 236 and also possibly
10   control the first and second shutter and filter wheels 228,
234. The instrumentation exclusive of the fiber sensor 200,
i.e., to the left of the proximal end of the bundle 202 is
discussed more completely by Bronk, *et al.*, Anal. Chem.
1995, Vol. 67, number 17, pp. 2750-2752.

15   The microsphere system 100 may be attached to the
distal end of the optical fiber bundle using a variety of
compatible processes. It is important that the microspheres
are located close to the end of the bundle. This ensures
that the light returning in each optical fiber
20   predominantly comes from only a single microsphere. This
feature is necessary to enable the interrogation of the
optical signature of individual microspheres to identify
reactions involving the microsphere's functionality and
also to decode the dye ratios contained in those
25   microspheres. The adhesion or affixing technique, however,
must not chemically insulate the microspheres from the
analyte.

Figs. 5A and 5B are micrographs of the distal end 212
of the bundle 202 illustrating the preferred technique for
30   attaching the microspheres 10 to the bundle 202. Wells 250
are formed at the center of each optical fiber 252 of the
bundle 202. As shown in Fig. 5B, the size of the wells 250
are coordinated with the size of the microspheres 10 so

that the microspheres 10 can be placed within the wells
250. Thus, each optical fiber 252 of the bundle 202 conveys
light from the single microsphere 10 contained in 5 its
well. Consequently, by imaging the end of the bundle 202
5   onto the CCD array 236, the optical signatures of the
microspheres 10 are individually interrogatable.

Fig. 6 illustrates how the microwells 250 are formed
and microspheres 10 placed in the wells. A 1 mm
hexagonally-packed imaging fiber contains approximately
10   20,600 individual optical fibers that have cores
approximately 3.7μm across (Part No. ET26 from Galileo
Fibers). Typically, the cores of each fiber are hexagonally
shaped as a result of glass hardness and drawing during
fiber fabrication. In some cases, the shape can be
15   circular, however.

In step 270, both the proximal and distal ends 212,214
of the fiber bundle 202 are successively polished on 12 μm,
9 μm, 3 μm, 1 μm, and 0.3 μm lapping films. Subsequently,
the ends can be inspected for scratches on an atomic force
20   microscope. In step 272, etching is performed on the distal
end 212 of the bundle 202. A solution of 0.2 grams NH_4F
(ammonium fluoride) with 600 μl dH_2O and 100 μl of HF
(hydrofluoric acid), 50% stock solution, may be used.   The
distal end 212 is etched in this solution for a specified
25   time, preferably approximately 80 seconds.

Upon removal from this solution, the bundle end is
immediately placed in deionized water to stop any further
etching in step 274. The fiber is then rinsed in running
tap water.   At this stage, sonication is preferably
30   performed for several minutes to remove any salt products
from the reaction. The fiber is then allowed to air dry.

The foregoing procedure produces wells by the anisotropic etching of the fiber cores 254 favorably with respect to the cladding 256 for each fiber of the bundle 202. The wells have approximately the diameter of the cores 254, 3.7 µm. This diameter is selected to be slightly larger than the diameters of the microspheres used, 3.1µm, in the example. The preferential etching occurs because the pure silica of the cores 254 etches faster in the presence of hydrofluoric acid than the germanium-doped silica claddings 256.

The microspheres are then placed in the wells 250 in step 276 according to a number of different techniques. The placement of the microspheres may be accomplished by dripping a solution containing the desired randomly mixed subpopulations of the microspheres over the distal end 212, sonicating the bundle to settle the microspheres in the wells, and allowing the microsphere solvent to evaporate. Alternatively, the subpopulations could be added serially to the bundle end. Microspheres 10 may then be fixed into the wells 250 by using a dilute solution of sulfonated Nafion that is dripped over the end. Upon solvent evaporation, a thin film of Nafion was formed over the microspheres which holds them in place. This approach is compatible for fixing microspheres for pH indication that carry FITC functionality. The resulting array of fixed microspheres retains its pH sensitivity due to the permeability of the sulfonated Nafion to hydrogen ions. This approach, however, can not be employed generically as Nafion is impermeable to most water soluble species. A similar approach can be employed with different polymers. For example, solutions of polyethylene glycol, polyacrylamide, or polyhydroxymethyl methacrylate (polyHEMA) can be used in place of Nafion, providing the requisite permeability to aqueous species.

An alternative fixation approach employs microsphere swelling to entrap each microsphere 10 in its corresponding microwell 250. In this approach, the microspheres are first distributed into the microwells 250 by sonicating the
5 microspheres suspended in a non-swelling solvent in the presence of the microwell array on the distal end 212. After placement into the microwells, the microspheres are subsequently exposed to an aqueous buffer in which they swell, thereby physically entrapping them, analogous to
10 muffins rising in a muffin tin.

One of the most common microsphere formations is tentagel, a styrene-polyethylene glycol co-polymer These microspheres are unswollen in nonpolar solvents such as hexane and swell approximately 20-40% in volume upon
15 exposure to a more polar or aqueous media. This approach is extremely desirable since it does not significantly compromise the diffusional or permeability properties of the microspheres themselves.

Figs. 7A and 7B show polymer coated microspheres 12 in
20 wells 250 after their initial placement and then after tapping and exposure to air pulses. Figs. 7A and 7B illustrate that there is no appreciable loss of microspheres from the wells due to mechanical agitation even without a specific fixing technique. This effect is
25 probably due to electrostatic forces between the microspheres and the optical fibers. These forces tend to bind the microspheres within the wells. Thus, in most environments, it may be unnecessary to use any chemical or mechanical fixation for the microspheres.

30 In alternative embodiments, additional encoding parameters can be added, such as microsphere size. If a number of sensors needed exceeds a few hundred, it is

possible to use microspheres of different sizes to expand the encoding dimensions of the microspheres. Optical fiber arrays can be fabricated containing pixels with different fiber diameters. With different diameters, the largest

5   wells can be filled with the largest microspheres and then moving onto progressively smaller microspheres in the smaller wells until all size wells are then filled. In this manner, the same dye ratio could be used to encode microspheres of different sizes thereby expanding the

10   number of different oligonucleotide sequences or chemical functionalities present in the array.

EXPERIMENTAL RESULTS
Enzyme-Based Sensor

Subpopulation A

15   Chemical functionality: Alkaline phosphatase
     Target substrate: fluorescein diphosphate (FDP)
     Reported dye ratio: 1:1 ratio of DiIC:TRC, where DiIC
is 1,1',3,3,3',3'-hexamethyl-indodicarbocyanine iodide and
TRC is Texas Red cadaverine

20   A range of ratios of light intensities are selected that are representative of the optical signature for the dye ratio of the subpopulation based on the quantum yield of the two dyes. The optical signature for this subpopulation is:

25   *iIC λ intensity-ave.DiIC background* =0.847 ± 0.23
     TRC λ intensity-ave.TRC background

Subpopulation B
Chemical functionality: B-Galactosidase;
     Target substrate = fluorescein di-B-galactopyranoside

30   (FDG)

Reporter dye ratio: 10:1 ratio of DiIC:TRC which translates to an optical signature of:

$DiIC \ \lambda \ intensity-ave. DiIC \ background = 4.456 \pm 1.27$

$TRC \ \lambda \ intensity-ave. TRC \ background$

Subpopulation C

Chemical functionality: B-glucuronidase

Target substrate = fluorescein di-B-D-glucuronide (FDGicu).

Reporter dye ratio: 1:10 ratio of DiIC:TRC, which translates to an optical signature of:

$DiIC \ A \ intensity-ave. \ DiIC \ background = 0.2136 + 0.03$

$TRC \ A \ intensity-ave. \ TRC \ background$

When the microsphere populations are in the presence of one or more of the substrates, the respective enzymes on the microspheres catalyze the breakdown of the substrates producing fluorescein which is fluorescent, emitting light at 530 nanometers when excited at 490 nm. The production of fluorescein localized to particular beads is then monitored. In this approach, the localization of fluorescein around the microspheres is increased by using a substrate solution of 90% glycerol and 10% substrate. The glycerol prevents the generated fluorescein from diffusing away from the microsphere reaction sites.

During the experiment, images in the encoded wavelengths are first taken. Since both DiIC and TRC are excited at 577 nm. Each microsphere's emissions at 670 nm, indicative of the presence of DiIC and 610 nm indicative of the presence of TRC were recorded using a 595 nm dichroic and an acquisition time of 5 seconds for the CCD 236. Next, the distal end 212 of the fiber bundle is placed in a buffer and another image taken while illuminating the beams

with 490 nm light. Emissions in the 530 nm fluorescein
wavelengths were recorded with a 505 nm dichroic. In this
case, a CCD acquisition time of one second was used. This
process provides a background normalizing image. The buffer
5   was removed and the fiber allowed to dry to avoid substrate
solution dilution.

The substrate solution is then introduced and CCD
images acquired every 30 seconds to a minute for 30 minutes
While illuminating the microspheres with 490 nm light and
10 collecting emissions in the 530 nm range. Fiber is then
placed back in the buffer solution and another background
image captured. Those beads that generate a signal
indicative of fluorescein production are decoded. Depending
in the ratio of the intensity of light from the two
15 reporter dyes, DiIC:TRC, the chemical functionality of the
optically active beads may be decoded according to the
following table.

0.617 – 1.08	alkaline phosphatase bead
3.188–5.725	β-galactosidase bead
0.183 – 0.243	β-glucunonidese bead

This process is then repeated for the remaining two
substrates.

Figs. 8A-8C are images generated by the CCD 236 when
the bead populations are exposed to fluorescein
25 diphosphate. Fig. 8A illustrates the signals from the
alkaline phosphatase microspheres when excited at 490 nm
and recording emissions at 530 nm, emissions at this
wavelength being indicative of fluorescein production. Fig.
8B shows the image captured by the CCD when the
30 microspheres are excited at 577 nm and emissions at 670 nm
are recorded. This wavelength is an encoding wavelength

indicative of the concentration of DiIC on the
microspheres. Finally, Fig. 8C shows the image when the
microspheres are excited with 577 nm light and emissions in
the 610 nm range are recorded being indicative of the
5   concentration of TRC in the microspheres.

        In a similar vein, Figs. 9A and 9B are images when the
microspheres are exposed to fluorescein d-β-galactosidase.
Fig. 9A shows emissions at 530 nm indicative of the
fluorescein production; and Fig. 9B shows light emitted at
10   the 670 nm range indicative of the presence of DiIC.

        These micrographs, Fig. 8A-8C and 9A-9B illustrate
fluorescein production around the microspheres may be
detected as an optical signature change indicative of
reactions involving the chemical functionality of the
15   microspheres. The micrographs also illustrate that the
optical signatures may be decoded to determine the chemical
functionalities on each microsphere.

Genosensor
        Three separate subpopulations of beads were used. In
20   subpopulation A, xrabbit antibodies (Ab) were affixed to
the surface of the microspheres; in subpopulation B, xgoat
antibodies were affixed to the microspheres; and in
subpopulation C, xmouse antibodies were affixed to the
microspheres. These three separate subpopulations were
25   identified using a DiIC:TRC encoding ratio similar to that
in the previously described experiment.

        For the first step of the experiment, images at the
 encoded wavelengths were captured using 577 nm excitation
and looking for emissions at 610 and 670 nm. After this
30   decoding, the fiber was placed in a buffer and an image
taken at 530 nm with 490 nm excitation. This provided a

background normalizing signal at the fluorescein emission wavelength. Next, the fiber was placed in rabbit IgG antigen (Ag) which is fluorescein labeled. Images were then captured every few minutes at the 530 nm emission

5   wavelength for fluorescein. Figs. 10A and 10B are micrographs showing the image captured by the CCD prior to and subsequent to exposure to a rabbit antigen, which clearly show reaction of the selected micropheres within the population.

10   Note, if the fluorescein background from the antigen solution is too high to see the antibody-antigen signal, the fiber bundle may be placed in a buffer. This removes the background florescence leaving only the Ab-Ag signal.

    While this invention has been particularly shown and

15   described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.